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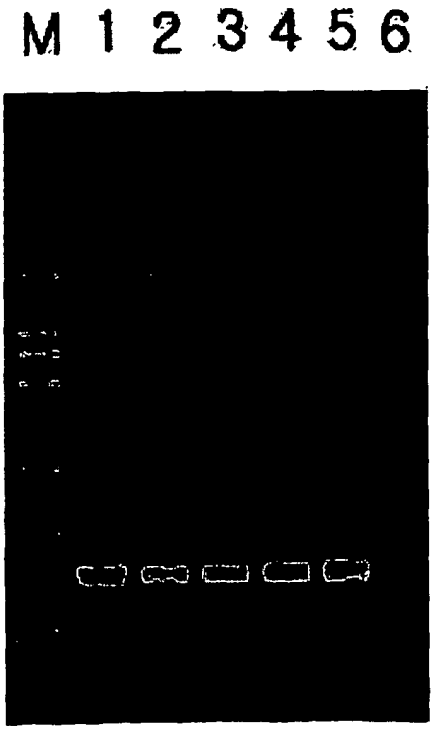
(54) Title: METHOD FOR PREPARING TRANSFORMED CUCUMIS VULGARIS

(57) Abstract: The present invention relates to a method of preparing a transformed Cucumis vulgaris using Acrobacterium tumefaciens, more particularly, to a method for preparing a transformed Cucumis vulgaris, which comprises the steps of: (a) inoculating a tissue of cotyledon or hypocotyl from Cucumis vulgaris with Agrobacterium tumefaciens harboring a suitable vector; and (b) regenerating the inoculated tissue of Cucumis vulgaris in a medium containing 6.0-1.0 mg/l of cytokinin and 0.2-0.0 mg/l of an auxin-based growth regulator.



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METHOD FOR PREPARING TRANSFORMED CUCUMIS VULGARIS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a method for preparing a transformed *Citrullus vulgaris*, more particularly, relates to a method of preparing a transformed *Citrullus vulgaris* using *Acrobacterium tumefaciens* and a transformed *Citrullus vulgaris* prepared therefrom.

Watervulgarisns (*Citrullus vulgaris* L.) belonging to Cucurbitaceous, are cultivated primarily in the North Africa and the Southeast Asia such as Korea, Japan, and China. The China has a cultivation area for Watervulgarisns which amounts to more than 60-70% of that of the whole world. Thus, the China has become a major target in export of watervulgarisn seed.

Watervulgarisns have been consumed chiefly from early summer to early autumn, but recently, as a result of developed technologies for cultivation using facilities starting from late in the 1980s, a production of watervulgarisns throughout the year has been permitted and consumption thereof has been made in all seasons. The total cultivation area of watervulgarisns is 40,000 ha which reaches to 11.9% of Korean vegetable cultivation area in 1999, and its continuous increase will be expected. The yield of watervulgarisns in raising outdoors is 260 thousand tons and that in raising by facilities is 670

thousand tons, and the total yield reaches to 930 thousand tons in 1999.

Recently, as a result of a rapid increase of the amount of cultivation and consumption of watervulgarisns, watervulgarisns maintain the third place of these records on the basis of annual total yield among various vegetables. In conjunction with WTO and import liberalization in farm products, the most of Korean domestic vegetables and fruits are considered not to be competitive internationally, but watervulgarisns are evaluated to have international competitiveness even after import liberalization.

A breeding of watervulgarisn has been made by routine and conventional breeding methods which has some problems: (a) having technological obstacles in enhancement of low-temperature pollen elongation, a construction of seed-gathering system by male sterilities, an improvement of sugar content, a development of seedless breeds, a an enrichment of storage and transport, a quality control of productive seeds and the like; (b) requirement for wider cultivation areas, higher cost and longer period resulting in incapable of satisfying consumer demand rapidly; and (c) difficulty in selection and fixation of cultivar with desirable traits.

Thus, the genetic engineering technologies have been requested to develop novel breeds of watervulgarisns.

For an improvement of plants by genetic manipulation, a

development of effective transformation technology is the most critical process. Such technology could solve the drawbacks that cannot be overcome by the conventional breeding technologies. Considering a development speed of the genetic engineering technologies and increase of genes available in agriculture, a development of transformation technology is crucial to future-oriented developments of agriculture for rearing high value-added breeds.

In addition, crops belonging to Cucurbitaceous have difficult problems for transformation. Especially, a transformation of watervulgarisns has not reported in Korea and foreign countries, and if any, the transformations thereof in published paper have a very low efficiency and are not repeatable (Choi P.S. et al., *Plant Cell Rep.*, 13(6):344-348(1994); Kim Y.S., *Mol. Cells*, 8(6)705-708(1998)). Moreover, there is no example of transformation of watervulgarisns useful in agriculture.

Therefore, the study of plant regeneration and transformation of watervulgarisns requires many efforts. The establishment of transformation of watervulgarisns is the most fundamental technology capable of accepting rapid altering demands of consumers and producing high-quality watervulgarisns with low cost. However, in case of watervulgarisns, there is no research or attempt to transform therefrom in Europe of America since they are cultivated in Korea, Japan, China and North Africa and so on and it has been reported that the breeds of

watervulgarisns are produced in Japan and China.

Throughout this application, various publications are referenced and citations are provided in parentheses. The disclosure of these publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

Under such situation, the present inventors have made intensive research to resolve the need in the art and as a result, we have completed the present invention by establishing a novel method for transformation of *Cucumis melo* such as a germination condition of seeds, a coculturing method with *Agrobacterium tumefaciens* and a unique composition of a regeneration medium. According to the present method, a preparation of a transformed *Cucumis melo* with *Agrobacterium tumefaciens* could be done more effectively in shorter time.

Accordingly, it is an object of this invention to provide a method for preparing a transformed *Cucumis vulgaris* using *Agrobacterium tumefaciens*.

It is another object of this invention to provide a transformed *Cucumis vulgaris prepared with the Agrobacterium tumefaciens*.

Other objects and advantages of the present invention

will become apparent from the detailed description to follow taken in conjunction with the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 represents a photograph demonstrating variation of regeneration ability of cotyledon from *Cucumis vulgaris* depending on germination time;

Fig. 2 represents a genetic map of binary vector pRD400 used in this invention;

Fig. 3 represents a photograph showing *in vitro* growth and rooting patterns of *Cucumis vulgaris* according to this invention; and

Fig. 4 represents a gel photograph showing the results of PCR elucidating transformed *Cucumis vulgaris* according to this invention.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of this invention, there is provided a method for preparing a transformed *Cucumis vulgaris*, which comprises the steps of: (a) inoculating a tissue of cotyledon or hypocotyl from *Cucumis vulgaris* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a genome of a cell from *Cucumis vulgaris* and contains the following sequences: (i) a replication origin operable in the cell from *Cucumis vulgaris*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis*

vulgaris; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence; and (b) regenerating the inoculated tissue of *Cucumis vulgaris* in a medium containing 6.0-1.0 mg/l of cytokinin and 0.2-0.0 mg/l of an auxin-based growth regulator.

In another aspect of this invention there is provided a method for preparing a transformed *Cucumis vulgaris*, which comprises the steps of: (a) germinating a seed of *Cucumis vulgaris* in a germination medium by dark culture for 2-6 days and successive light culture for 12-30 hours; (b) inoculating a tissue of cotyledon from *Cucumis vulgaris* formed by germination with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a genome of a cell of cotyledon from *Cucumis vulgaris* and contains the following sequences: (i) a replication origin operable in the cell from *Cucumis vulgaris*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis vulgaris*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence; and (c) regenerating the inoculated tissue from *Cucumis vulgaris* in a medium containing 6.0-1.0 mg/l of cytokinin and 0.1-0.0 mg/l of an auxin-based growth regulator.

The present invention will be described in more detail as follows:

I. Preparation of Starting Material for Transformation

The preferred explant for transformation includes leaf, stem and petiole, but not limited to. The explant may be obtained from several plant organs and most preferably from seed. It is preferred that the seed is sterilized with sterilizing agent such as chlorine and chlorides (e.g., sodium hypochloride) before use.

II. Seed Germination

According to a preferred embodiment of this invention, the medium for seed germination comprises nutrient basal medium such as MS, B5, LS, N6 and White's, energy source and vitamins, but not limited to. Sugars are useful as energy source and sucrose is the most preferable. It is preferred that vitamins for seed germination include nicotine, thiamine and pyridoxine. In addition, the medium for seed germination in this invention may further contain MES (2-(N-Morpholino) ethanesulfonic acid Monohydrate) as buffering agent for pH change and agar as solid support. The medium is unlikely to contain plant growth regulators.

The period for dark culture is critical for seed germination. According to a preferred embodiment, for seed germination, the dark culture is performed for 2-6 days and the light culture for 12-30 hr. More preferably, the period for dark culture is 3-5 days and that for light culture is 20-28 hr. Most preferably, the period for dark culture is 4 days and that for light culture is 24 hr.

Regeneration of explant is largely dependent on the period for dark culture as demonstrated in Example. The illumination intensity for light culture is usually 3000-5000 lux. It is preferred that seed germination is performed at the temperature of 25±1℃.

III. Preparation of Plant Tissue for Transformation

In this invention, the explant for transformation includes any tissue derived from seed germinated. It is preferred to use cotyledon and hypocotyl and the most preferred is cotyledon. It is advantageous to remove growth point completely from cotyledon as explant.

IV. Inoculation with *Agrobacterium tumefaciens*

Transformation of cells derived from *Cucumis vulgaris* is carried out with *Agrobacterium tumefaciens* harboring Ti plasmid (Depicker, A. et al., Plant cell transformation by *Agrobacterium* plasmids. In Genetic Engineering of Plants, Plenum Press, New York (1983)). More preferably, binary vector system such as pBin19, pRD400 and pRD320 is used for transformation (An, G. et al., Binary vectors" In Plant Gene Res. Manual, Martinus Nijhoff Publisher, New York(1986)).

The binary vector useful in this invention carries: (i) a promoter capable of forming RNA operating in the cell from *Cucumis vulgaris*; (ii) a structural gene operably linked to the promoter; and (iii) a polyadenylation signal

sequence. In addition to this, it is preferred that the vector carries antibiotics-resistance gene as selective marker, e.g. carbenicllin, kanamycin, spectinomycin and hygromcin. The vector may alternatively further carry a gene coding for reporter molecule (for example, luciferase and β-glucuronidase). Examples of the promoter used in the binary vector include but not limited to Cauliflower Mosaic Virus 35S promoter, 1′ promoter, 2′ promoter and promoter nopaline synthetase (nos) promoter. The structural gene in the present vector may be determined depending on traits of interest. Exemplified structural gene may include but not limited to genes for herbicide resistance (e.g. glyphosate, sulfonylurea), viral resistance, vermin resistance (e.g., Bt gene), resistance to environmental extremes (e.g. draught, high or low temperature, high salt conc.), improvement in qualities (e.g. increasing sugar content, retardation of ripening), exogenous protein production useful as drug (EGF, antigen or antibody to various diseases, insulin) or cosmetic raw material (e.g. albumin, antibiotic peptide).

Inoculation of the explant with *Agrobacterium tumefaciens* involves procedures known in the art. Most preferably, the inoculation involves dissecting cotyledon with growth point removed and immersing these sections in culture of *Agrobacterium tumefaciens* to coculture, thereby inoculating the cotyledon with *Agrobacterium tumefaciens*. The *Agrobacterium tumefaciens* is infected through

dissected side. Such method is developed to shorten a coculturing time remarkably. This effect may be accomplished using only two sections of cotyledon. In the present invention, the period for coculturing is 1 hr-5 min, more preferably 20-7 min.

Conventionally, an inoculation of cells derived from *Cucumis vulgaris* has been done using cotyledon with several hurts and culture of *Agrobacterium tumefaciens* (Choi P.S. et al., *Plant Cell Rep.*, 13(6):344-348(1994)). This conventional method has been very likely to bring about necrosis of plant tissue because of long-term coculturing of cotyledon with several hurts. However, according to this invention, such disadvantage may be completely overcome.

Preferably, acetosyringone is employed in the coculturing medium to promote infection of *Agrobacterium tumefaciens* into explant cell.

V. Regeneration

It is necessary that explant tissue, which is transformed with *Agrobacterium tumefaciens*, be regenerated in regeneration medium with strictly controlled ingredients and quantities thereof. The regeneration medium of this invention may contain nutrient basal medium such as MS, B5, LS, N6 and White's, energy source and vitamins, but not limited to. Sugars are useful as energy source and sucrose is the most preferable. It is preferred

that vitamins in regeneration medium include nicotine, thiamine and pyridoxine. In addition, the regeneration medium may further contain MES (2-(N-Morpholino) ethanesulfonic acid Monohydrate) as buffering agent for pH change and agar as solid support.

The medium must contain plant growth regulators. Cytokinin as plant growth regulator may include but not limited to 6-benzylaminopurine (BAP), kinetin, zeatin and isopentyladenosine and BAP is the most preferable cytokinin. The auxin (for example, 1-naphthalene acetic acid, indole acetic acid, (2,4-dichlorophenoxy) acetic acid) is contained in the regeneration medium of this invention, which is thought to be distinguished feature in consideration of conventional medium.

Preferably, the amount of cytokinin in the regeneration medium is 4.0-1.5 mg/l, the most preferably 2.0 mg/l. The amount of the auxin is preferably 0.02-0 mg/l, the most preferably 0 mg m/l.

According to a preferred embodiment of this invention, the medium further contains antibiotics (e.g. carbenicllin, kanamycin, spectinomycin or hygromcin) for selection of transformed explant.

Most preferably, the culture in regeneration medium is performed under the following conditions: 25±1℃; 16 hr:8 hr (light culture : dark culture). The period necessary for culture varies widely, preferably about 3-6 weeks.

Culturing according to the conditions described above

allows successfully a regeneration of shoots through callus formation from the transformed explant of *Cucumis vulgaris* on the medium.

VI. Rooting

The transformed *Cucumis vulgaris* plantlet is finally produced on rooting medium by rooting of regenerated shoots. The rooting medium of this invention may contain nutrient basal medium such as MS, B5, LS, N6 and White's, energy source and vitamins, but not limited to. Sugars are useful as energy source and sucrose is the most preferable. It is preferred that vitamins in the rooting medium include nicotine, thiamine and pyridoxine. In addition, the rooting medium may further contain MES (2-(N-Morpholino) ethanesulfonic acid Monohydrate) as buffering agent for pH change and agar as solid support.

As plant growth regulator, auxin is predominantly employed in the rooting medium. The auxin useful includes 1-naphthalene acetic acid, indole acetic acid and (2,4-dichlorophenoxy) acetic acid, and the most preferable is indole acetic acid.

Preferably, antibiotics to select transformed *Cucumis vulgaris* are not contained in the rooting medium of this invention.

VII. Confirmation of Transformation

The transformed *Cucumis vulgaris* produced according to

the present invention may be confirmed using procedures known in the art. For example, using DNA sample from tissue of transformed *Cucumis vulgaris*, PCR is carried out to elucidate exogenous gene incorporated into a genome of *Cucumis vulgaris* transformed. Alternatively, Northern or Southern Blotting may be performed for confirming the transformation as described in Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.(1989).

In a yet another aspect of this invention, there is provided a method for preparing a transformed *Cucumis vulgaris*, which comprises the steps of: (a) germinating a seed of *Cucumis vulgaris* in a germination medium by dark culture for 3-5 days and light culture for 20-28 hours, in which the germination medium contains a nutrient basal medium selected from the group consisting of MS, B5, LS, N6 and White's; (ii) an energy source; and (iii) vitamins; (b) inoculating the tissue of *Cucumis vulgaris* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a genome of a cell of cotyledon from *Cucumis vulgaris* forming by germination and contains the following sequences: (i) a replication origin operable in the cell from *Cucumis vulgaris*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis vulgaris*; (iii) a structural gene operably linked to the promoter; (iv) a polyadenylation signal

sequence; and (v) an antibiotics-resistance gene as a selective marker; and (c) regenerating the inoculated tissue from *Cucumis vulgaris* on a medium containing (i) 4.0-1.5 mg/l of cytokinin selected from the group consisting of 6-benzylaminopurine, kinetin, zeatin and isopentyladenosine; (ii) a nutrient basal medium selected from the group consisting of MS, B5, LS, N6 and White's; (iii) a sugar as an energy source; and (iv) vitamins; and (d) rooting shoots regenerated in the regeneration step on a rooting medium containing (i) an auxin-based growth regulator selected from the group consisting of 1-naphthalene acetic acid, indole acetic acid and (2,4-dichlorophenoxy) acetic acid; (ii) a nutrient basal medium selected from the group consisting of MS, B5, LS, N6 and White's; (iii) a sugar as an energy source; and (iv) vitamins.

In another aspect of the present invention, there is provided a transformed *Cucumis vulgaris* prepared by the methods of this invention described above.

The method of this invention, which is developed for producing a transformed *Cucumis vulgaris*, as exemplified and demonstrated in Examples below, exhibits much higher transformation and regeneration efficiency with shorter period for manipulation, giving rise to production of transformed *Cucumis vulgaris* having desirable traits with

higher reproducibility.

The following specific examples are intended to be illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

EXMPLE 1: Preparing of Explants

4 cultivars of *Cucumis vulgaris* (Apollo, SacheolKul, SuperKeumcheon and Keumbo), which have been developed in Korea, were employed in regeneration and transformation experiments. Seed coats from seeds of 4 cultivars kept at 4℃ were removed with physical method, sterilized with occasional agitation in 4% NaOCl solution for 30 min and washed 4 times with DW. The sterilized seeds were placed on germination media containing 1/2 MSMS, 1.0% sucrose and 0.6% agar and then cultured to germinate seed for 4 days at 25±1℃ under dark culture condition. Thereafter, the resulting cotyledons or hypocotyls were used as samples.

EXAMPLE 2: Regeneration of Explant Tissue

To prepare a suitable medium composition for regeneration of cotyledon or hypocotyl, the cotyledon and hypocotyl obtained above were placed on 4 types of media

containing ingredients described in Table 1, and cultured for 4 weeks at 25±1℃ under the condition of 16 hrs/8 hrs (light/dark), followed by examination of regeneration rate and average number of regenerated shoots.

The regeneration rate was calculated from percentage of ratio of the number of regenerated section to total number of section placed and the average number of regenerated shoot was calculated from percentage of ratio of the number of regenerated shoot to the number of regenerated section. The results are summarized in Table 2. The basal media containing MSB5 (Murashige & Skoog medium including Gamborg B5 vitamins), 500 ㎎/ℓ of MES(2-(N-Morpholino) ethanesulfonic acid Monohydrate), 3% sucrose and 0.6% agar were employed for regeneration.

TABLE 1

	Medium 1	Medium 2	Medium 3	Medium 4
NAA (mg/l)	0	0	0.1	0.1
BAP (mg/l)	2	4	2	4
Salt	MS[1)]	MS	MS	MS
Vitamins	B5[2)]	B5	B5	B5

1) MS: Murashige & Skoog medium, and [2)]B5: nicotinic acid, thiamine-HCl and pyridoxine-HCl contained

TABLE 2

Regeneration rate and average No. of shoot depending on cultivar and medium composition									
Var	Ex-plant	BAP/NAA (mg/l)							
		2.0/0.0		4.0/0.0		2.0/0.1		4.0/0.1	
		Reg [5]	Shoot [6]	Reg	Shoot	Reg	Shoot	Reg	Shoot
A[1]	Cot[7]	53.0	1.33	40.0	1.13	33.0	0.73	20.0	0.47
	Hyp[8]	0.0	0.00	0.0	0.00	0.0	0.00	6.0	0.22
B[2]	Cot	50.0	1.10	20.0	0.30	25.0	0.50	30.0	0.35
	Hyp	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
C[3]	Cot	50.0	1.00	30.0	0.40	15.0	0.30	10.0	0.15
	Hyp	0.0	0.00	0.0	0.50	0.0	0.20	0.0	0.50
D[4]	Cot	30.0	0.55	10.0	0.10	5.0	0.05	0.0	0.00
	Hyp	0.0	0.00	0.0	0.00	5.0	0.05	5.0	0.05

[1]SuperKeumcheon, [2]SacheolKul, [3]Apollo, [4]Kumbo, [5]cotyledon, [6]hytpcotyl, [7]regeneration rate, and [8]average number of shoots regenerated

As shown in Table 2, the regeneration rates in cotyledon were revealed in the wide range of 0-50%. In the case of using auxin-based growth regulator (NAA), the regeneration rates were found to decrease largely. Apollo cultivar treated with 0.1 mg/l of NAA showed 38-50% decrease of regeneration rate compared to that not treated with NAA, which also was observed in SuperKeumcheon and Keumbo. While SacheolKul treated with 2.0 mg/l of BAP and 0.1 mg/l of NAA showed the reduced regeneration rate, 25%, SacheolKul treated with 4.0 mg/l of BAP and 0.1 mg/l of NAA showed 10% increase of regeneration rate compared to that not treated with NAA.

2.0/0.0 of BAP/NAA gave rise to excellent results in view of the average number of regenerated shoots and the

increase of NAA concentration resulted in the decrease of number of regenerated shoots.

As to hypocotyl, the regeneration rate was much lower, shoots were rarely regenerated, and callus formed were finally dead.

As revealed from the results, as explant for transformation of *Cucumis vulgaris*, cotyledon is the most preferable, the medium optimal for transformation contains BAP (2 mg/l) without auxin.

EXAMPLE 3: Regeneration Ability of Cotyledon Depending on Germination Time

With the cotyledon showing the most excellent regeneration ability evaluated in Example 2, a relationship between a harvest time of cotyledon and a regeneration ability was examined as follows: First, seeds of SuperKeumcheon were treated in the same manner as described in Example 1, then cultured for 1, 2, 3, 4 and 5 days, respectively, at 25℃±1℃ under dark condition and additionally cultured at 25℃±1℃ and 4,000 lux for 1 day under light condition. Thereafter, the cotyledons were taken and placed on the medium containing 2 mg/l of BAP representing the most outstanding regeneration rate in Example 2, followed by culturing for 4 weeks to measure the regeneration rate and the number or regenerated shoots.

It was elucidated that the regeneration ability of cotyledon varied differently depending on dark culture

period. Especially, it was observed that the regeneration rate of the cotyledon dark-cultured for 5 days was decreased to less than half that observed in maximal regeneration and the cotyledon dark-cultured for less than 1 day showed few regenerated shoots. The harvest time of cotyledon for shoot regeneration was favorable in dark culture for 2-4 days. Especially, it was easier to remove a growth point from cotyledon dark-cultured for 4 days and the cotyledon was in good shape (Fig. 1). In Fig. 1, panels A, B, C and D represent regeneration patterns of cotyledon of *Cucumis vulgaris* dark-cultured for 1 day, 2 days, 4 days and 5 days, respectively. As shown in Fig. 1, the group cultured for 1-2 days had some deviation among sections and showed to generate a multitude of shoots, and the group cultured for 4 days gave rise to 2-3 regenerated shoots which were derived from callus generated in dissected region. In addition, the group dark-cultured for 5 days showed the decrease by less than half in regeneration ability.

Therefore, the most suitable germination period is 4 day-dark culture.

EXAMPLE 4: Transformation of Explant

To transform cotyledon of *Cucumis vulgaris*, firstly, seeds of SuperKeumcheon were treated in the same manner as Example 1, dark-cultured for 4 days followed by light-cultured for 1 day and cotyledons with growth point cut

were then taken.

Agrobacterium tumefaciens (*Agrobacterium tumefaciens* GV3101(Mp90); *Plant-cell-rep.*, 15(11)799-803(1996)) transformed with binary vector pRD400 (Fig. 2) were cultured in super broth (37 g/l brain heart infusion broth(Difco), 0.2% sucrose, pH 5.6) containing 200 μM of acetosyringone for 18 hrs, the resulting medium was diluted with Infection broth containing 1 mg/l of 2,4-D and ingredients of Table 3 and DMSO solution to the ratio of 1:37:2. In Fig. 2, LB and RB represent left and right border of T-DNA, respectively, MCS represents multiple cloning site, Tnos and Pnos represent termination sequence and promoter sequence of nos, respectively, and nptII represents neomycin phosphotransferase II sequence.

Thereafter, the cotyledon with cut side in terminal was immersed in the mixed solution and cocultured for 10 min to inoculate the cotyledon with *Agrobacterium tumefaciens* through the cut side. This inoculation method may permit not only to shorten period for transformation of *Cucumis vulgaris* but also to prevent necrosis of explant happened usually during coculture cotyledon with several hurts.

After coculturing for 10 min, the cotyledon was placed in a coculturing medium containing 2 mg/l of BAP (4.04 g/l MSB5, 3.0% sucrose, 0.5 g/l MES, 0.6% agar, pH 5.6) and cultured at 4,000 lux under 16 hour-light culture condition at 25℃±1℃ for 2 days. Cultured cotyledon was placed in the regeneration medium of Table 3 containing

solely 500 mg/l of carbenicillin and pre-cultured at 25℃± 1℃ for 7 days to induce generation of shoots. Then, the shoots induced were cultured in the selection medium of Table 3 containing 200 mg/l of kanamycin for 4 weeks followed by fixing the regenerated shoots selected with kanamycin.

Fixed regenerated shoots were subcultured in the rooting medium of Table 3 containing carbenicillin and kanamycin and after 2-3 weeks, the rooting shoots, which were considered to be transformed, were selected.

TABLE 3

Compositions of media for transformation					
Ingredient	Germ. Med.[1)]	Inoculation broth	Reg. Med.[2)]	Sel. Med.[3)]	Root Med.[4)]
Salt	MS	MS	MS	MS	MS
Vitamins	B5	B5	B5	B5	B5
Sucrose(g/l)	10	20	20	30	30
MES(mg/l)	500	500	500	500	500
Phytagel (g/l)	6 g (agar)	-	4.0	4.0	2.0
2,4-D(mg/l)	-	1	-	-	-
BAP(mg/l)	-	-	2	2	-
IAA(mg/l)	-	-	-	-	0.1
Others	-	AS[5)]	CBC[6)]	CBC KM[7)]	-

1)germination medium, 2)regeneration medium, 3)selection medium, 4)rooting medium, 5)acetosyringone (200 μM), 6)carbenicillin (500 mg/l), and 7)carbenicillin (500 mg/l) and kanamycin (200 mg/l)

Cotyledon of SuperKeumcheon cocultured with *Agrobacterium tumefaciens* began to form a multiple of callus after 10 days, and to form a multiple of shoots

after 2-3 weeks. However, all shoots formed were not found to be transformed and in the case of subculturing the shoots regenerated in selection medium or rooting medium, most shoots with no transformation were likely to be whitened or withered. On the other hand, the transformed shoots were found to form roots after about 2-3 weeks and the rooting was also observed even in several subculture (Fig. 3). In Fig. 3, panel A is a photograph of transformed *Cucumis vulgaris* which was grown *in vitro* (means that *Cucumis vulgaris* transformant can fix roots normally and proliferate on media containing antibiotics), and panel B is a photograph showing rooting patterns on selection media containing antibiotics.

EXAMPLE 5: Confirmation of Transformant

The shoots rooted, which were considered to be transformed, were confirmed in view of transformation by PCR analysis as follows: Firstly, Genomic DNA for PCR analysis was obtained from transformant selected in Example 4 using the method described by Edwards K., et al. (*Nucleic Acids Research*, 19: 1349(1991)).

The primers for PCR were designed to have complementary sequence to *nptII* gene (encoding neomycin phosphotransferase II) of the vector in *Agrobacterium tumefaciens*: forward primer, 5'-GAT GGA GTG CAC GCA GGT-3' and reverse primer, 5'-TCA GAA GAA CTC GTC AAG-3'. In the PCR, the mixture consisting of 2.5 μl of 10x reaction

buffer (Boeringher anheim, containing 2.5 mM Mg^{2+}, pH 7.5), 2.0 µl of mixture of dNTPs (10 nM), 1 µl of template DNA and 0.25 µl of Taq polymerase (5 U/µl) per 25 µl of total reaction solution was used. The PCR was performed in such a manner that pre-denaturation at 94℃ for 1 minute and denaturation at 94℃ for 1 minute were done consecutively, and total 35 cycles were done in which each cycle is composed of denaturation at 94℃ for 1 minute, annealing at 55℃ for 1 minute and extension at 72℃ for 2 minute, followed by final extension at 72℃ for 10 minute. The PCR product was subject to electrophoresis on 1.0% agarose gel (Fig. 4). In Fig. 4, lane M shows 1 kb ladder, lanes 1-5 show PCR products of *Cucumis vulgaris* transformed according to this invention and lane 6 shows PCR products of *Cucumis vulgaris* not transformed.

As shown in Fig. 4, in the PCR using genomic DNA from transformed *Cucumis vulgaris* selected in Example 4, about 0.8 kb of *npt*II gene was amplified. Thus, it is confirmed that the transformed *Cucumis vulgaris* selected in Example 4 is transformed according to this invention having exogenous gene on the genomic DNA.

In conclusion, according to the present invention, novel *Cucumis vulgaris* with desirable traits can be obtained with higher regeneration and transformation rate. In addition to this, the method of the present invention can shorten a transformation time remarkably.

What is claimed is:

1. A method for preparing a transformed *Cucumis vulgaris*, which comprises the steps of:

(a) inoculating a tissue of cotyledon or hypocotyl from *Cucumis vulgaris* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a genome of a cell from *Cucumis vulgaris* and contains the following sequences:

(i) a replication origin operable in the cell from *Cucumis vulgaris*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis vulgaris*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence; and

(b) regenerating the inoculated tissue of *Cucumis vulgaris* in a medium containing 6.0-1.0 mg/l of cytokinin and 0.2-0.0 mg/l of an auxin-based growth regulator.

2. A method for preparing a transformed *Cucumis vulgaris*, which comprises the steps of:

(a) germinating a seed of *Cucumis vulgaris* in a germination medium by dark culture for 2-6 days and successive light culture for 12-30 hours;

(b) inoculating a tissue of cotyledon from *Cucumis vulgaris* formed by germination with *Agrobacterium tumefaciens* harboring a vector, in which the vector is

capable of inserting into a genome of a cell of cotyledon from *Cucumis vulgaris* and contains the following sequences:

(i) a replication origin operable in the cell from *Cucumis vulgaris*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis vulgaris*; (iii) a structural gene operably linked to the promoter; and (iv) a polyadenylation signal sequence; and

(c) regenerating the inoculated tissue from *Cucumis vulgaris* in a medium containing 6.0-1.0 mg/l of cytokinin and 0.1-0.0 mg/l of an auxin-based growth regulator.

3. The method according to claim 1 or claim 2, wherein the cytokinin is selected from the group consisting of 6-benzylaminopurine, kinetin, zeatin and isopentyladenosine.

4. The method according to claim 1 or 2, wherein the auxin-based growth regulator is selected from the group consisting of 1-naphthalene acetic acid, indole acetic acid and (2,4-dichlorophenoxy) acetic acid.

5. The method according to claim 1 or 2, wherein the amount of cytokinin is 4.0-1.5 mg/l and the amount of auxin-based growth regulator is 0.02-0.0 mg/l.

6. The method according to claim 5, wherein the amount of

cytokinin is 2.0 mg/l and the amount of auxin-based growth regulator is 0.0 mg/l.

7. The method according to claim 2, wherein the step of germinating is performed by dark culture for 3-4 days and successive light culture for 16-24 hours.

8. The method according to claim 1, wherein the cell is derived from a cotyledon.

9. The method according to claim 8, wherein the step of inoculating a tissue from *Cucumis vulgaris* with *Agrobacterium tumefaciens* is executed by immersing a section of the cotyledon into a culture of *Agrobacterium tumefaciens* and coculturing for 5-60 min.

10. The method according to claim 9, wherein the step of inoculating a tissue from *Cucumis vulgaris* with *Agrobacterium tumefaciens* is executed by immersing a section of the cotyledon into a culture of *Agrobacterium tumefaciens* and coculturing for 7-20 min.

11. The method according to claim 10, wherein the step of inoculating a tissue from *Cucumis vulgaris* with *Agrobacterium tumefaciens* is executed by dissecting a cotyledon into two sections and immersing the sections into a culture of *Agrobacterium tumefaciens* and

coculturing for 7-20 min.

12. A method for preparing a transformed *Cucumis vulgaris*, which comprises the steps of:

(a) germinating a seed of *Cucumis vulgaris* in a germination medium by dark culture for 3-5 days and light culture for 20-28 hours, in which the germination medium contains a nutrient basal medium selected from the group consisting of MS, B5, LS, N6 and White's; (ii) an energy source; and (iii) vitamins;

(b) inoculating the tissue of *Cucumis vulgaris* with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of inserting into a genome of a cell of cotyledon from *Cucumis vulgaris* forming by germination and contains the following sequences:

(i) a replication origin operable in the cell from *Cucumis vulgaris*; (ii) a promoter capable of promoting a transcription in the cell from *Cucumis vulgaris*; (iii) a structural gene operably linked to the promoter; (iv) a polyadenylation signal sequence; and (v) an antibiotics-resistance gene as a selective marker; and

(c) regenerating the inoculated tissue from *Cucumis vulgaris* on a medium containing (i) 4.0-1.5 mg/l of cytokinin selected from the group consisting of 6-benzylaminopurine, kinetin, zeatin and

isopentyladenosine; (ii) a nutrient basal medium selected from the group consisting of MS, B5, LS, N6 and White's; (iii) a sugar as an energy source; and (iv) vitamins; and

(d) rooting shoots regenerated in the regeneration step on a rooting medium containing (i) an auxin-based growth regulator selected from the group consisting of 1-naphthalene acetic acid, indole acetic acid and (2,4-dichlorophenoxy) acetic acid; (ii) a nutrient basal medium selected from the group consisting of MS, B5, LS, N6 and White's; (iii) a sugar as an energy source; and (iv) vitamins.

13. A transformed *Cucumis vulgaris* prepared by the method according to claims 1, 2 or 12.

Fig. 1

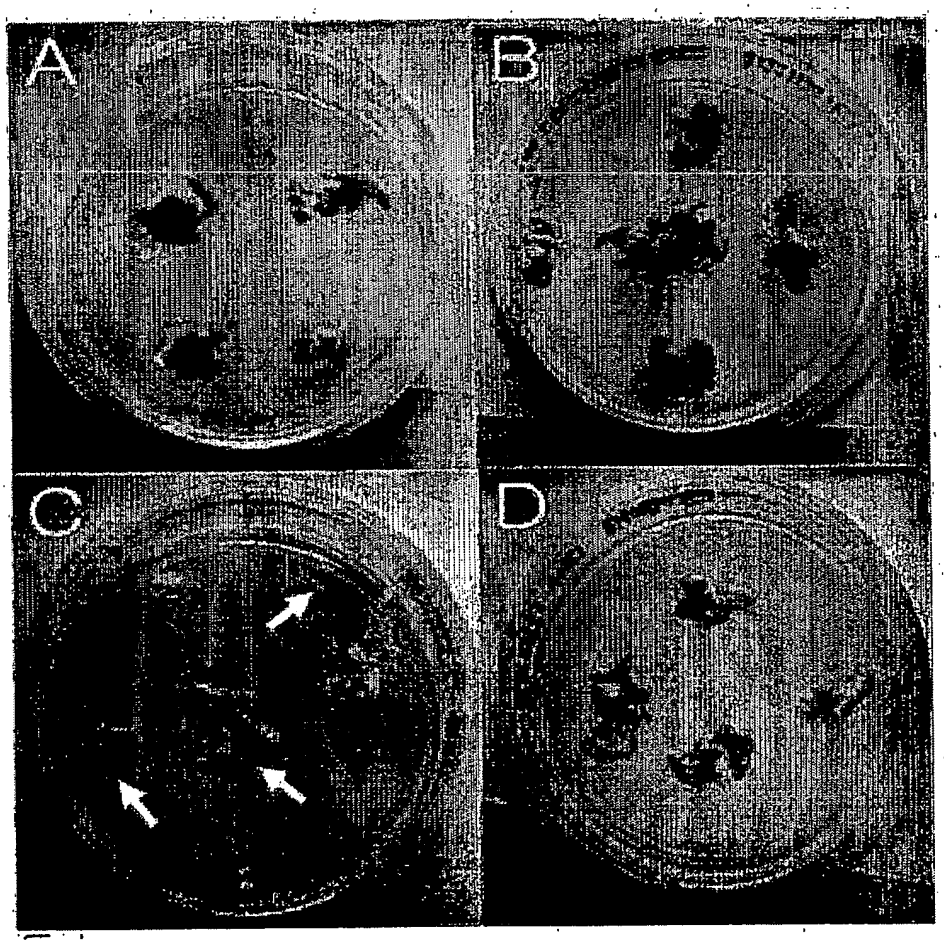


Fig. 2

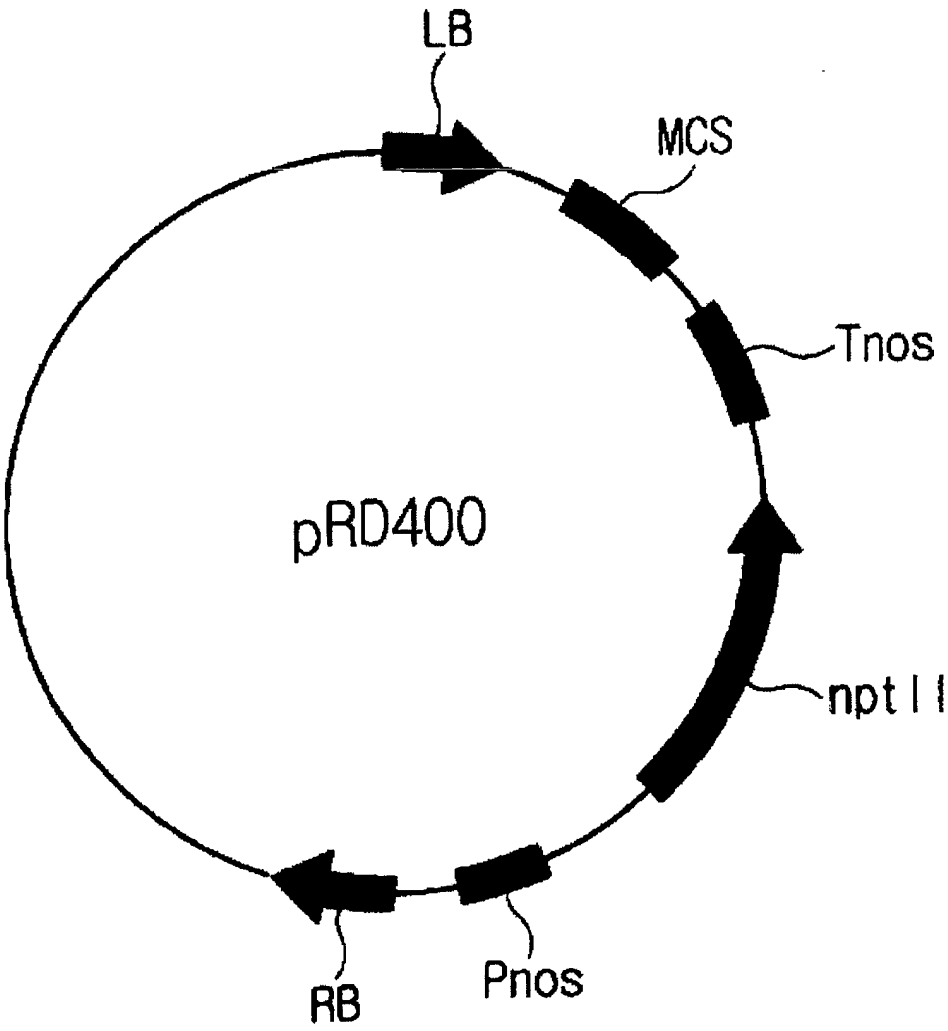


Fig. 3

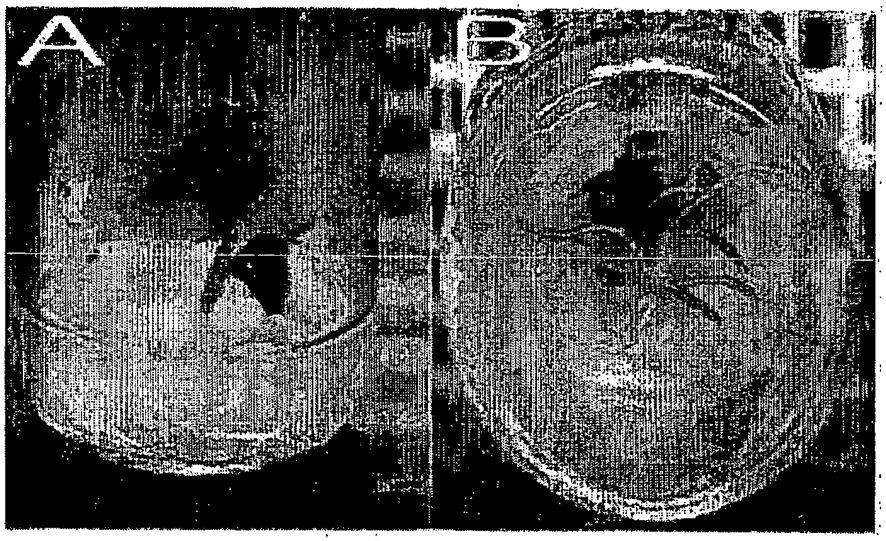
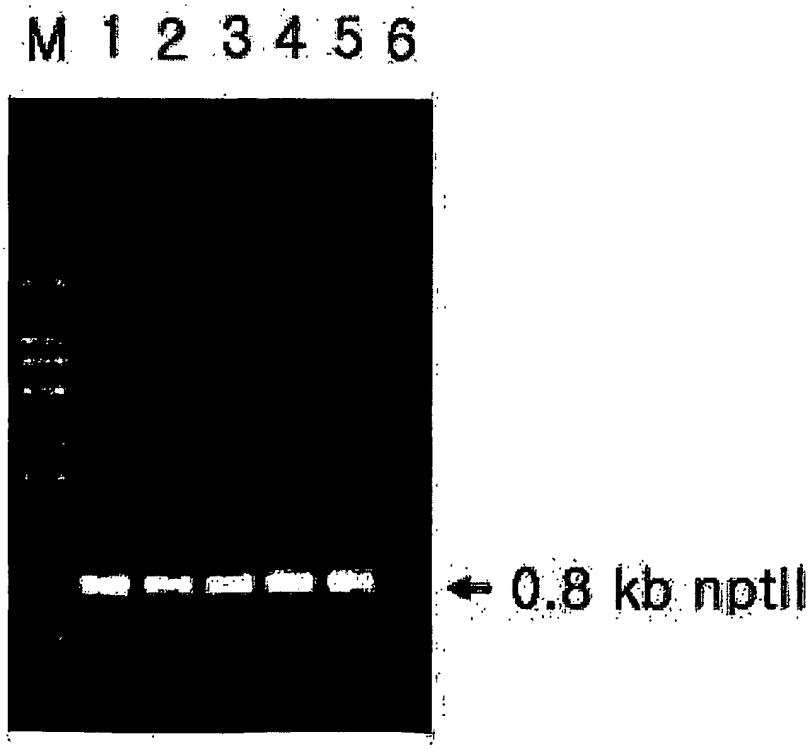


Fig. 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 02/01463

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C12N 15/82, 15/29, A01H 4/00, 3/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C12N 15/82, 15/29, A01H 4/00, 3/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHOI, P.S. et al. Genetic transformation and plant regeneration of watermelon using Agrobacterium tumefaciens. Plant Cell Reports, 1994, Vol. 13, No. 6, pages 344-348 *the whole document.*	1-3,7,8,12,13
A	WO 95/02056 A2 (THE UPJOHN COMPANY) 19 January 1995 (19.01.95) *claims 1, 7-13, 17.*	1,2,12,13
A	WO 90/03725 A1 (THE UPJOHN COMPANY) 19 April 1990 (19.04.90) *example 1; pages 10-13, 18, 19; claims 1-16.*	1-13
A	US 5614467 A (FRANKENBERBER et al.) 25 March 1997 (25.03.97) *column 2, lines 9, 10; claims.*	1,2,4,12,13

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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12 November 2002 (12.11.2002)	4 December 2002 (04.12.2002)
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US	A	5614467	25-03-1997			none	
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				CN	A	1041784	02-05-1990
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